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The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

Report on the ICCVAM / NICEATM Expert Panel Meeting on In Vitro ER/AR Assays

William Stokes, D.V.M., D.A.C.L.A.M.,
Barbara Shane, Ph.D., D.A.B.T.,
Raymond Tice, Ph.D., Christina Inhof, M.S.P.H.
NICEATM, NIEHS, Research Triangle Park, NC

U.S. EPA EDVMS Meeting
Washington DC
July 24, 2002

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Expert Panel Meeting

- **This presentation summarizes selected conclusions and recommendations of an independent Expert Panel as discussed at their meeting on May 21-22, 2002.**
  - **The complete final meeting report may contain minor changes. The Expert Panel Meeting Report will be published in August, 2002; an electronic version will be available at http://iccvam.niehs.nih**
- **The opinions presented here are those of the independent panel and do not necessarily reflect the positions of the NIEHS or ICCVAM**

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Outline

- Background
- Expert Panel members and charge
- Selected Panel recommendations
  - General recommendations
  - Assay-specific recommendations

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ICCVAM Evaluation of Estrogen Receptor (ER) and Androgen Receptor (AR) *In Vitro* Methods

2000
- U.S. EPA Request to ICCVAM for independent scientific peer review to:
  - Assess the validation status of these *in vitro* methods
  - Develop minimum performance criteria that could be used to define acceptable *in vitro* assays

2001
- *Federal Register* (3/21/01) request for:
  - Data and information on ER and AR methods
  - Nomination of experts for Panel
- Background Review Documents (BRDs) prepared
  - No standardized methods with completed validation studies located

2002
- *Federal Register* Notice (4/5/02) announces□□□□:
  - Expert Panel Meeting, May 21-22, 2002
  - Availability of BRDs
  - Request for public comments

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Acknowledgements

Preparation of BRDs

Bradley Blackard*
Christina Inhof*
Barbara Shane*
Raymond Tice*
Gail Tudor**
Errol Zeiger*

Support of Expert Panel Meeting

Bradley Blackard*
Sue Brenzel*
Loretta Frye***
Christina Inhof*
Linda Litchfield*
Debbie McCarley***
Vicki Wilson, EPA
Steven Myers*
Michael Paris*
Barbara Shane*
Judy Strickland*
Raymond Tice*
Errol Zeiger*

*ILS, Inc, Research Triangle Park, NC
**University of North Carolina, Chapel Hill, NC
*** NIEHS, Research Triangle Park, NC

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ICCVAM Endocrine Disruptor Working Group (EDWG)

- ATSDR
  Stephanie Miles-Richardson
- CPSC
  Marilyn Wind (Co-Chair)
- FDA
  Michael Bolger
  Paul Brown
  Kenneth Delclos
  Charles Eirkson
  Suzanne Fitzpatrick
  David Hattan (Co-Chair)
  Abby Jacobs
  Devaraya Jagannath
  Alexander Jordan
  David Moore
  Charles Parfitt
  Leonard Schechtman
  Melvin Stratmeyer
  Thomas Umbreit
- DOI
  Timothy Gross
- EPA
  Angela Auletta
  Sally Perreault-Darney
  Karen Hamernik
  Richard Hill
- NIEHS
  Jerry Heindel
  Retha Newbold
  William Stokes
  Julius Thigpen
- NIOSH
  Eisuke Murono
- OSHA
  Surrender Ahir

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Expert Panel Members

ER Binding Assay Primary Reviewers

- George Daston (Panel Chair), *Procter & Gamble*
- Nira Ben-Jonathan, *University of Cincinnati*
- Robert Combes, *FRAME, United Kingdom*
- John Harbell, *Institute for In Vitro Sciences, Inc.*
- Stephen Safe, *Texas A & M University*
- James Wittliff, *University of Louisville*
- Walter Piegorsch (Biostatistician), *University South Carolina*

ER Transcriptional Activation Assay Primary Reviewers

- John Stegman (Chair), *Woods Hole Oceanographic Inst.*
- Grantley Charles, *Dow Chemical Company*
- Ellen Mihaich, *Rhodia, Inc.*
- Thomas Wiese, *Tulane and Xavier Universities*
- James Yager, *Johns Hopkins School of Public Health*
- Timothy Zacharewski, *Michigan State University*
- Shyamal Peddada (Biostatistician), *NIEHS*

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Expert Panel Members

AR Binding Assay Primary Reviewers

- Terry Brown (Chair), *Johns Hopkins University*
- Thomas Gasiewicz, *University of Rochester Medical Center*
- Tohru Inoue, *National Institute of Health Sciences, Japan*
- Bernard Robaire, *McGill University, Canada*
- Anne Marie Vinggaard, *Danish Veterinary and Food Administration, Denmark*
- Walter Piegorsch (Biostatistician), *University of South Carolina*

AR Transcriptional Activation Assay Primary Reviewers

- Elizabeth Wilson (Chair), *UNC-Chapel Hill*
- Kevin Gaido, *CIIT Centers for Health Research*
- William Kelce, *Pharmacia Corporation*
- Shyamal Peddada (Biostatistician), *NIEHS*

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Background Review Documents (BRDs)

- ER Binding Assays
- ER Transcriptional Activation Assays
- AR Binding Assays
- AR Transcriptional Activation Assays

Each BRD contains:

- Available protocols
- A review of the procedural components for each type of assay
- Proposed minimum procedural standards
- Proposed assays for validation
- Proposed substances (chemicals) for future validation studies

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Charge to the Expert Panel

Review BRDs and provide conclusions and recommendations on the following:

1. Assays that should be considered for further evaluation in validation studies, and their relative priority
2. Adequacy of the proposed minimum procedural standards for each of the 4 types of assays
3. Adequacy of available protocols for assays recommended for validation studies
4. Adequacy and appropriateness of the substances recommended for validation studies

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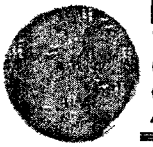

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ER Binding Assays

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ER Binding Assay BRD Database

- 14 Assays
  - Uterine cytosol: mouse, rabbit, rat
  - Cell cytosol: human breast cancer (MCF-7)
  - Whole cells: human breast cancer (MCF-7)
  - Purified ER: human (h) ERα, hERα + fluorescence polarization, rat (r) ERα, rERβ
  - GST constructs: anole (a), chicken (c), mouse (m), rainbow trout (rt), h ER(def)
- 635 substances tested in one or more assays
- Comparative performance and reliability: Limited data; however, assays using purified ER (human or rat α or β) appeared more sensitive than the rat uterine cytosol (RUC) assay

ER Binding

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Panel Recommendations: ER Binding Assay Development/Validation

- Highest priority should be development of assays using purified recombinant protein receptors (human or rat ERα and β).
- Development of an exogenous method for metabolic activation is desirable but not essential.
- Consideration should be given to non-radioactive methods (e.g., fluorescent polarization).

ER Binding

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Recommended (Selected) Minimum Procedural Standards for All Assays

- For binding assays
  - the dissociation constant (K_d) of the reference estrogen/androgen determined with each assay
  - the concurrent positive control should have a binding affinity 2-3 orders of magnitude below that of the reference estrogen/ androgen and is tested at multiple concentrations
  - sodium molybdate and a cocktail of protease inhibitors added to protect the ER/AR from degradation
  - For binding assays, substances that bind but do not bring about a 50% reduction in ER/AR binding should be classified as "equivocal".
- Test substances prepared in water, 95-100% ethanol, or DMSO (in order of preference)
- Solvent controls included in each assay

All Assays

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Recommended (Selected) Minimum Procedural Standards for All Assays

- The limit concentration is 1 millimolar (mM); solubility characteristics of each test substance must be taken into consideration
- Concentration range of test substances span at least 7 orders of magnitude and consist of at least 7 different concentrations spaced one order of magnitude apart
- Triplicate measurements performed at each concentration tested

Recommended (Selected) Minimum Procedural Standards for All Assays

- For TA assays
  - the stability of cell lines with a stably transfected reporter must be monitored
  - an assessment of cellular cytotoxicity should be included to define the upper limit for test substance concentrations
  - For transient transfection methods, a constitutive reporter gene assay must be included to assess the efficiency of transfection
- For an assay to be acceptable, the reference estrogen/ androgen and/or positive control responses must be consistent with historical data

Recommended (Selected) Minimum Procedural Standards for All Assays

- For TA assays, a suitable nonlinear regression model such as the Hill equation should be used to estimate the potency (EC_{50} or IC_{50} values) and slope of the concentration-response curve with a 95% confidence interval
- Classification of a test substance as 'positive' should be based on the use of statistical models pertinent to the characteristics of the assay
- Replicate studies are not essential but questionable data confirmed by re-testing
- All studies requiring animals as tissue sources approved by an IACUC
- The assays should be conducted following Good Laboratory Practice guidelines

All Assays

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Recommended (Selected) Minimum Procedural Standards for ER Binding Assays

- The reference estrogen is hexa-tritium labeled 17β-estradiol.
- Dextran-coated charcoal the preferred procedure for separating bound from free labeled 17β-estradiol.

ER Binding

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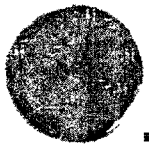
Adequacy of ER Binding Protocols

- The U.S. EPA RUC protocol, revised to include the minimum procedural standards, was recommended as a template for other ER binding assays.
- All other ER binding protocols should incorporate the minimum procedural standards.

Proposed Test Substances for ER Binding Validation Studies

- BRD Recommendations:
  - 33 substances with ER binding assay data
    - 3 (10%) negative substances
    - 5 substances at each of 6 orders (log spaced) of relative binding activity values (from <0.001 to >10).
- Panel Recommendations:
  - Accept the BRD list.
  - Increase the proportion of negative substances to at least 25% to enhance assessment of assay specificity.



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ER Transcriptional Activation Assays

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ER TA Assay BRD Database

- 95 Assays
  - 63 mammalian cell TA assays
    - 9 human cell lines
    - 3 nonhuman mammalian cell lines
    - ER, ERα, ERβ (human, mouse, rat) receptors
    - Chloramphenicol acetyltransferase or luciferase reporter gene responses
  - 22 yeast (*S. cerevisiae)* TA assays
    - hER, hERα, hERβ, mER, rtER receptors
    - β-galactosidase reporter gene response
  - 10 mammalian cell proliferation assays
    - 4 human cell lines
- 698 substances tested in one or more assays
- Data inadequate for an assessment of comparative performance and reliability

ER TA

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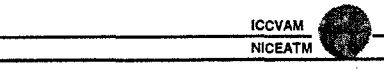
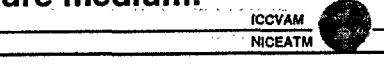
Panel Recommendations: ER TA Assay Development/Validation

- No specific assay recommended.
- Recommended pre-validation study on stably vs transiently transfected cell line with hERα expression vector, using a reporter construct with multiple vitellogenin estrogen response elements (vit-ERE) + luciferase (include cell line with endogenous hERα).
- Development of an exogenous metabolic activation system was not recommended.



Recommended (Selected) Minimum Procedural Standards for ER TA Assays

- The reference estrogen (positive control for agonist studies) should be 17β estradiol.
- A relatively weak estrogenic agonist (e.g., estriol) should be included as an additional positive control for agonist experiments.
- The positive control for antagonist studies should be a relatively active antagonist (e.g., ICI 182,780).
- Classification of a test substance as 'positive' for agonist or antagonist activity should be based on the generation of a concentration response curve.
- To ensure that a positive agonist response is due to receptor-mediated activity, the test substance could be re-tested with ICI 182,780 (the candidate ER antagonist) present in the culture medium.



Adequacy of ER TA Protocols

- Additional detail needs to be added to the luciferase activity protocol.
- Add standardized procedures for counting cells, determining % confluency, and seeding of plates.
- Add assay acceptability criteria to ensure each experiment is performed in the absence of estrogenic contamination.
- Assess and describe the metabolic capabilities of the cell lines selected for assays.
- All protocols should incorporate the minimum procedural standards.

Proposed Test Substances for ER TA Validation Studies

- BRD Recommendations:
  - Agonist assays
    - 31 substances with ER TA agonist assay data
      - 5 (16%) negative substances
      - 26 substances classified as weak to potent agonists
  - Antagonist assays
    - 21 substances with ER TA antagonist assay data
      - 4 (19%) negative substances
      - 17 substances classified as weak to potent antagonists
- Panel Recommendations:
  - Accept the BRD list.
  - The same substances should be used for ER binding and ER TA validation studies.

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AR Binding Assays

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AR Binding Assay BRD Database

- 11 Assays
  - ➢ Tissue cytosol: calf uterus; rat prostate, epdidymis
  - ➢ Cell cytosol: hAR transfected monkey kidney cells (COS-1), human cancer cells (MCF-7, LnCAP)
  - ➢ Whole cells: human genital fibroblasts (HGF)
  - ➢ AR transfected cells: hAR or rtAR transfected COS-1 cells
  - ➢ Purified AR: hARα
- 109 substances tested in one or more assays
- Data inadequate for an assessment of comparative performance and reliability

AR Binding

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Panel Recommendations: AR Binding Assay Development/Validation

- An assay using purified recombinant hAR should be developed and validated.
- As an alternative to using the recombinant hAR (patent protected), a recombinant AR from other primates could be considered.
- Inclusion of an exogenous method for metabolic activation not recommended.
- Consideration should be given to non-radioactive methods (e.g., fluorescent polarization).

Recommended (Selected) Minimum Procedural Standards for AR Binding Assays

- The recommended reference androgen for recombinant protein-based assays (i.e., cell-free assays), where metabolism of DHT would not occur, is 5α-dihydrotestosterone (DHT).
- Due to its high affinity, lack of metabolism, and low non-specific protein binding, the recommended reference androgen for most other assays is R1881.
- However, as R1881 binds to the progesterone receptor (PR), binding assays based on cells or tissues that contain this receptor should include triamcinolone acetonide to block its binding to the PR.

Recommended (Selected) Minimum Procedural Standards for AR Binding Assays

- Alternatively, mibolerone, which has a low affinity for PR, is appropriate as the reference androgen for such assays.
- The concurrent positive control (e.g., cyproterone acetate) should have a binding affinity 2-3 orders of magnitude below that of the reference androgen and must be tested at multiple doses.

Adequacy of AR Binding Protocols

- No standardized, acceptable protocol was included in the BRD.
- The standardized protocol for the U.S. EPA rat prostate cytosol (RPC) assay requires additional information.
- All protocols should incorporate the minimum procedural standards.

Proposed Test Substances for AR Binding Validation Studies

- BRD Recommendations:
  - 31 substances with AR binding assay data
    - 3 (10%) negative substances
    - 4-5 substances at each of 6 orders (log spaced) of relative binding activity values (from <0.001 to >10).
- Panel Recommendations:
  - Accept the BRD list.
  - The number of negative substances should be increased.
  - Bicalutamide, hydroxyflutamide, finasteride should be included.
  - The same substances should be used in the validation of both AR binding and AR TA assays.

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AR Transcriptional Activation Assays

AR TA Assay BRD Database

- 17 Assays
  - ➢ 15 Mammalian cell TA assays
    - 6 human cell lines
    - 3 nonhuman vertebrate cell lines
    - AR (human, mouse, rainbow trout) receptors
    - Chloramphenicol acetyltransferase or luciferase reporter gene responses
  - ➢ 1 Yeast (*S. cerevisiae)* assay
    - AR (human) receptor
    - β-galactosidase reporter gene response
  - ➢ 1 Mammalian cell proliferation assay
- 147 substances tested in one or more assays
- Data inadequate for an assessment of comparative performance and reliability

Panel Recommendations: AR TA Assay Development/Validation

- Develop an assay using cells (e.g., MDA-MB-453) that contain an endogenous AR and which are stably transfected with adenovirus containing MMTV-Luc reporter.
- However, the development of a transiently AR transfection assay would probably be more sensitive than an endogenous AR assay, if patents do not limit.
- Inclusion of an exogenous method for metabolic activation not recommended.

Recommended (Selected) Minimum Procedural Standards for AR TA Assays

- The reference androgen should be R1881.
- The transcriptional activation response of the reference androgen must be demonstrated by a full concentration response curve.
- The positive control for agonist studies should be an androgen that is two orders of magnitude less active than R1881.
- The positive control for antagonist studies should be a relatively active substance (e.g., hydroxyflutamide).
- Serum-free and phenol red-free media should be used rather than charcoal stripped serum.

Adequacy of AR TA Protocols

- Protocols were considered inadequate with regard to level of details provided; information on performance and reliability should be provided.
- All protocols should incorporate the minimum procedural standards.

Proposed Test Substances for AR TA Validation Studies

- BRD Recommendations:
  - Agonist assays
    - 28 substances with AR TA agonist assay data
      - 10 (36%) negative substances
      - 18 classified as weak to potent agonists
  - Antagonist assays
    - 25 substances with AR TA antagonist assay data
      - 4 (16%) negative substances
      - 21 classified as weak to potent antagonists
- Panel Recommendations:
  - A list of ~20 substances for pre-validation agonism and antagonism studies.
  - Add substances that might interfere with luciferase production (e.g., by inhibiting RNA or protein synthesis).

AR TA

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Other (Selected) Panel Recommendations

- Metabolic activation
  - The effect of metabolism on the estrogenic/ androgenic activity of the reference substances should be evaluated, including whether metabolites have activity.
  - The extent that metabolic activation contributes to the formation of endocrine active substances should be assessed.
  - If important, appropriate metabolic activation systems for in vitro assays should be developed.
- Chemicals selected for *in vivo* endocrine disruptor validation studies should be tested in these *in vitro* assays.

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Other (Selected) Panel Recommendations

- A central repository of coded chemicals with verified purity should be made available for future validation studies to ensure comparability of data.
- Appropriate agencies should investigate the status of patents and licenses and provide guidance to the scientific community on the development of assays using proprietary materials.
- A sequential testing strategy should be evaluated, where a positive *in vitro* assay might preclude subsequent testing in a different binding/TA assay.

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